

CHAPTER 2.3.4.

AVIAN INFLUENZA (INFECTION WITH AVIAN INFLUENZA VIRUSES)

SUMMARY

Influenza A is caused by specified viruses that are members of the family Orthomyxoviridae and placed in the genus influenza virus A. There are three influenza genera – A, B and C; only influenza A viruses are known to infect birds. Diagnosis is by isolation of the virus or by detection and characterisation of fragments of its genome. This is because infections in birds can give rise to a wide variety of clinical signs that may vary according to the host, strain of virus, the host's immune status, presence of any secondary exacerbating organisms and environmental conditions.

Identification of the agent: *Suspensions in antibiotic solution of oropharyngeal and cloacal swabs (or faeces) taken from live birds, or of faeces and pooled samples of organs from dead birds, are inoculated into the allantoic cavity of 9- to 11-day-old embryonated chicken eggs. The eggs are incubated at 37°C (range 35–39°C) for 2–7 days. The allantoic fluid of any eggs containing dead or dying embryos during the incubation and all eggs at the end of the incubation period are tested for the presence of haemagglutinating activity. The presence of influenza A virus can be confirmed by an immunodiffusion test between concentrated virus and an antiserum to the nucleocapsid and/or matrix antigens, both of which are common to all influenza A viruses, or by real-time reverse-transcription polymerase chain reaction (RT-PCR) on the allantoic fluids. Isolation in embryos has recently been replaced under certain circumstances by direct detection in samples, of one or more segments of the influenza A genome using real-time RT-PCR or other validated molecular techniques.*

For serological subtyping of the virus, a reference laboratory should conduct haemagglutination and neuraminidase inhibition tests against a battery of polyclonal or monospecific antisera to each of the 16 haemagglutinin (H1–16) and 9 neuraminidase (N1–9) subtypes of influenza A virus. Alternatively the genome of specific H and N subtypes is identified using RNA detection technologies with subtype specific primers and probes (e.g. real-time RT-PCR) or sequencing and phylogenetic analysis.

As the general term “highly pathogenic avian influenza” and the historical term ‘fowl plague’ refer to infection with highly pathogenic strains of influenza A virus, it is necessary to assess the pathogenicity of Influenza A virus isolates for domestic poultry. Although all naturally occurring “highly pathogenic avian influenza” strains isolated to date have been either of the H5 or H7 subtype, most H5 or H7 isolates have been of low pathogenicity. The methods used for the determination of strain virulence for birds have evolved over recent years with a greater understanding of the molecular basis of pathogenicity, but still primarily involve the intravenous inoculation of a minimum of eight susceptible 4- to 8-week-old chickens with infectious virus; strains are considered to be highly pathogenic if they cause more than 75% mortality within 10 days, or inoculation of 10 susceptible 6-week-old chickens resulting in an intravenous pathogenicity index (IVPI) of greater than 1.2. Characterisation of suspected highly pathogenic strains of the virus should be conducted in a virus-secure biocontainment laboratory. Regardless of their pathogenicity for chickens, H5 or H7 viruses with a HA0 cleavage site amino acid sequence similar to any of those that have been observed in highly pathogenic viruses are considered influenza A viruses with high pathogenicity. H5 and H7 isolates that are not highly pathogenic for chickens and do not have an HA0 cleavage site amino acid sequence similar to any of those that have been observed in highly pathogenic viruses are considered to have low pathogenicity. For the purposes of the OIE Terrestrial Animal Health Code, avian influenza is notifiable to OIE and is defined as an infection of poultry caused by any influenza A virus with high pathogenicity (HPAI) and by H5 and H7 subtypes

with low pathogenicity (H5/H7 LPAI). Influenza A viruses with high pathogenicity in birds other than poultry, including wild birds, are also notifiable. Low pathogenicity non-H5 and non-H7 influenza A viruses (i.e. H1–4, H6 and H8–16) are not defined as avian influenza and are not notifiable.

Serological tests: As all influenza A viruses have antigenically similar nucleoprotein and matrix antigens, these are preferred targets of influenza A group serological methods. Agar gel immunodiffusion tests can be used to detect antibodies to these antigens. Concentrated virus preparations containing either or both type of antigens are used in such tests. Not all species of birds develop demonstrable precipitating antibodies. Enzyme-linked immunosorbent assays have been used to detect antibodies to influenza A type-specific antigens in either species-dependent (indirect) or species-independent (competitive) test formats. Haemagglutination inhibition tests have also been employed in routine diagnostic serology, but it is possible that this technique may miss some particular infections because the haemagglutinin is subtype specific.

Requirements for vaccines: Historically, in most countries, vaccines specifically designed to contain or prevent HPAI were banned or discouraged by government agencies because they may interfere with stamping-out control policies. The first use of vaccination in an avian influenza eradication programme was against H5/H7 LPAI. The programmes used inactivated oil-emulsion vaccines with the same haemagglutinin and neuraminidase subtypes as the circulating field virus, and infected flocks were identified by detection of virus or antibodies against the virus in non-vaccinated sentinel birds. During the 1990s the prophylactic use of inactivated oil-emulsion vaccines was employed in Mexico and Pakistan to control widespread outbreaks of HPAI and H5/H7 LPAI, and a recombinant fowl poxvirus vaccine expressing the homologous HA gene was also used in Mexico, El Salvador and Guatemala. During the 1999–2001 outbreak of H7 LPAI in Italy, an inactivated vaccine was used with the same (i.e. homologous) haemagglutinin subtype to the field virus, but with a different (i.e. heterologous) neuraminidase. This allowed the serological differentiation of non-infected vaccinated birds from vaccinated birds infected with the field virus and ultimately resulted in eradication of the field virus. Prophylactic use of H5 and H7 vaccines has been practised in parts of Italy, aimed at preventing H5/H7 LPAI infections, and several countries in Asia, Africa and the Middle East as an aid in controlling H5N1 HPAI virus infections. HPAI viruses should not be used as the seed virus for production of vaccine.

If HPAI is used in challenge studies, the facility should meet the OIE requirements for containment Group 4 pathogens.

A. INTRODUCTION

Influenza in birds is caused by infection with viruses of the family *Orthomyxoviridae* placed in the genus *influenzavirus A*. Influenza A viruses are the only orthomyxoviruses known to naturally affect birds. Many species of birds have been shown to be susceptible to infection with influenza A viruses; aquatic birds form a major reservoir of these viruses, and the overwhelming majority of isolates have been of low pathogenicity (low virulence) for chickens and turkeys. Influenza A viruses have antigenically related nucleocapsid and matrix proteins, but are classified into subtypes on the basis of their haemagglutinin (H) and neuraminidase (N) antigens (World Health Organization Expert Committee, 1980). At present, 16 H subtypes (H1–H16) and 9 N subtypes (N1–N9) are recognised with proposed new subtypes (H17, H18) for influenza A viruses from bats in Guatemala (Swayne *et al.*, 2013; Tong *et al.*, 2012; 2013). To date, naturally occurring highly pathogenic influenza A viruses that produce acute clinical disease in chickens, turkeys and other birds of economic importance have been associated only with the H5 and H7 subtypes. Most viruses of the H5 and H7 subtype isolated from birds have been of low pathogenicity for poultry. As there is the risk of a H5 or H7 virus of low pathogenicity (H5/H7 low pathogenicity avian influenza [LPAI]) becoming highly pathogenic by mutation, all H5/H7 LPAI viruses from poultry are notifiable to OIE. In addition, all high pathogenicity viruses from poultry and other birds, including wild birds, are notifiable to the OIE.

Currently, the OIE *Terrestrial Animal Health Code (Terrestrial Code)* defines “avian influenza” as an infection of **poultry** caused by any influenza A virus with high pathogenicity (HPAI), and by H5 and H7 subtypes with low pathogenicity (H5/H7 LPAI). In previous versions of the *Terrestrial Code* and *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (Terrestrial Manual)*, HPAI and H5/H7 LPAI viruses were termed “notifiable avian influenza” viruses, but because of inconsistencies with usage of the term “notifiable” with other diseases in the *Terrestrial Code*, the terms “notifiable avian influenza”, “high pathogenicity notifiable avian influenza” and “low pathogenicity notifiable avian influenza” have been removed from the *Terrestrial Code* and the *Terrestrial Manual*. To avoid confusion with the scientific use of “avian influenza”, which began in 1955, in this *Terrestrial Manual* the

terms HPAI, H5/H7 LPAI and influenza A will be used. The latter indicates any influenza virus from birds that is H1–H16.

Depending on the species, age and type of bird, specific characteristics of the viral strain involved, and on environmental factors, the highly pathogenic disease, in fully susceptible birds, may vary from one of sudden death with no overt clinical signs, to a more characteristic disease with variable clinical presentations including respiratory signs, such as ocular and nasal discharges, coughing, snicking and dyspnoea, swelling of the sinuses and/or head, apathy, reduced vocalisation, marked reduction in feed and water intake, cyanosis of the unfeathered skin, wattles and comb, incoordination and nervous signs and diarrhoea. In laying birds, additional clinical features include a marked drop in egg production, usually accompanied by an increase in numbers of poor quality eggs. Typically, high morbidity is accompanied by high and rapidly escalating unexplained mortality. However, none of these signs can be considered pathognomonic. In certain host species such as Pekin ducks some HPAI viruses do not necessarily provoke significant clinical disease. In addition, influenza A viruses of low pathogenicity which normally cause only a mild or no clinical disease, may in certain circumstances produce a spectrum of clinical signs, the severity of which may approach that of HPAI, particularly if exacerbating infections and/or adverse environmental conditions are present. Confirmatory diagnosis of the disease, therefore, depends on the isolation or detection of the causal virus and the demonstration that it fulfils one of the defined criteria described in section B.2. Testing sera from suspect birds using antibody detection methods may supplement diagnosis, but these methods are not suitable for a definitive identification. Diagnosis for official control purposes is established on the basis of agreed official criteria for pathogenicity according to *in-vivo* tests or to molecular determinants (i.e. the presence of a cleavage site of the haemagglutinin precursor protein HA0 consistent with HPAI virus) and haemagglutinin subtyping. These definitions evolve as scientific knowledge of the disease increases.

HPAI and H5/H7 LPAI are subject to official control. The viruses that cause HPAI and H5/H7 LPAI have the potential to spread from the laboratory if adequate levels of biosecurity and biosafety are not in place. Avian influenza viruses are classed at a minimum in Risk Group 2 for human and animal infection and should be handled with appropriate measures as described in Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*. Biocontainment measure should be determined by risk analysis as described in Chapter 1.1.4. The measures required may vary among the subtypes, with higher level containment (e.g. Risk group 3 or 4) being indicated for H5/H7 LPAI and HPAI viruses. Countries lacking access to such a specialised national or regional laboratory should send specimens to an OIE Reference Laboratory.

B. DIAGNOSTIC TECHNIQUES

Table 1. Test methods available for the diagnosis of avian influenza and their purpose

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Agent identification¹						
Virus isolation	+	+++	+	+++	+	–
Antigen detection	+	+	+	+	+	–
Real-time RT-PCR	++	+++	++	+++	++	–
Detection of immune response						
AGID	+ (Influenza A)	+ (Influenza A)	++ (Influenza A)	+ (convalescent)	++ (Influenza A)	++ (Influenza A)

¹ A combination of agent identification methods applied on the same clinical sample is recommended.

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
HI	+++ (H5 or H7)	++ (H5 or H7)	+++ (H5 or H7)	++ (convalescent)	+++ (H5 or H7)	+++ (H5 or H7)
ELISA	+	+	++	+ (convalescent)	++	++

Key: +++ = recommended method; ++ = suitable method; + = may be used in some situations, but cost, reliability, or other factors severely limits its application; – = not appropriate for this purpose.
 Although not all of the tests listed as category +++ or ++ have undergone formal validation, their routine nature and the fact that they have been used widely without dubious results, makes them acceptable.
 RT-PCR = reverse-transcription polymerase chain reaction; AGID = agar gel immunodiffusion;
 HI = haemagglutination inhibition test; ELISA = enzyme-linked immunosorbent assay.

1. Identification of the agent

1.1. Virus isolation

Virus isolation is the “gold standard” but laborious and time insensitive, used primarily for diagnosis of first clinical case and to obtain virus isolated for further laboratory analysis.

Samples taken from dead birds should include intestinal contents (faeces) or cloacal swabs and oropharyngeal swabs. Samples from trachea, lungs, air sacs, intestine, spleen, kidney, brain, liver and heart should also be collected and processed either separately or as a pool.

Samples from live birds should include both oropharyngeal and cloacal swabs. To avoid harming them, swabbing of small delicate birds should be done with the use of especially small swabs that are usually commercially available and intended for use in human paediatrics. Where these are not available, the collection of fresh faeces may serve as an alternative. Similar swab samples can be pooled (i.e. cloacal swabs with cloacal swabs, oropharyngeal swabs with oropharyngeal swabs), and most commonly pooling of 5 or 11 samples, but specific swab types should be used (Spackman *et al.*, 2013).

The samples should be placed in isotonic phosphate-buffered saline (PBS), pH 7.0–7.4 with antibiotics or a solution containing protein and antibiotics. The antibiotics can be varied according to local conditions, but could be, for example, penicillin (2000 units/ml), streptomycin (2 mg/ml), gentamycin (50 µg/ml) and mycostatin (1000 units/ml) for tissues and oropharyngeal swabs, but at five-fold higher concentrations for faeces and cloacal swabs. It is important to readjust the pH of the solution to pH 7.0–7.4 following the addition of the antibiotics. It is recommended that a solution for transport of the swabs should contain protein to stabilise the virus (e.g. brain–heart infusion, up to 5% [v/v] cattle serum, 0.5% [w/v] bovine albumen or similar commercially available transport media). Faeces and finely minced tissues should be prepared as 10–20% (w/v) suspensions in the antibiotic solution. Suspensions should be processed as soon as possible after incubation for 1–2 hours at room temperature. When immediate processing is impracticable, samples may be stored at 4°C for up to 4 days. For prolonged storage, diagnostic samples and isolates should be kept at –80°C. Repeated freezing and thawing should be avoided.

The preferred method of growing influenza A viruses is by the inoculation of specific pathogen free (SPF) embryonated chicken eggs, or specific antibody negative (SAN) eggs. The supernatant fluids of faeces or tissue suspensions obtained through clarification by centrifugation at 1000 *g* are inoculated into the allantoic sac of three to five embryonated SPF or SAN chicken eggs of 9–11 days' incubation. The eggs are incubated at 37°C (range 35–39°C) for 2–7 days. Eggs containing dead or dying embryos as they arise, and all eggs remaining at the end of the incubation period, should first be chilled to 4°C for 4 hours or overnight, and the allantoic fluids should then be recovered and tested with a screening test (such as haemagglutination [HA] test), influenza A type-specific test (such as agar gel immunodiffusion test [AGID] or solid-phase antigen-capture enzyme-linked immunosorbent assays [ELISA]) or influenza A subtype-specific test (such as haemagglutination inhibition [HI] and neuraminidase inhibition [NI] tests) or a molecular test to detect influenza A specific nucleic acid signatures (such as real-time reverse transcription polymerase chain reaction [RT-PCR] test) as described later (see Section B.3.2). Detection of HA activity, in bacteria-free amnio-allantoic fluids

verified by microbiological assay, indicates a high probability of the presence of an influenza A virus or of an avian paramyxovirus. Fluids that give a negative reaction should be passaged into at least one further batch of eggs.

The presence of influenza A virus can be confirmed in AGID tests by demonstrating the presence of the nucleocapsid or matrix antigens, both of which are common to all influenza A viruses (see Section B.3.1). The antigens may be prepared by concentrating the virus from infective allantoic fluid or extracting the infected chorioallantoic membranes; these are tested against known positive antisera. Virus may be concentrated from infective allantoic fluid by ultracentrifugation, or by precipitation under acid conditions. The latter method consists of the addition of 1.0 M HCl to infective allantoic fluid until it is approximately pH 4.0. The mixture is placed in an ice bath for 1 hour and then clarified by centrifugation at 1000 *g* at 4°C. The supernatant fluid is discarded. The virus concentrates are resuspended in glycylsarcosyl buffer: this consists of 1% (w/v) sodium lauroyl sarcosinate buffered to pH 9.0 with 0.5 M glycine. These concentrates contain both nucleocapsid and matrix polypeptides.

Preparations of nucleocapsid-rich antigen can also be obtained from chorioallantoic membranes for use in the AGID test (Beard, 1970). This method involves removal of the chorioallantoic membranes from infected eggs that have allantoic fluids with HA activity. The membranes are then homogenised or ground to a paste. This is subjected to three freeze–thaw cycles, followed by centrifugation at 1000 *g* for 10 minutes. The pellet is discarded and the supernatant is used as an antigen following treatment with 0.1% formalin.

Use of the AGID test to demonstrate nucleocapsid or matrix antigens is a satisfactory way to indicate the presence of influenza A virus in amniotic fluid, but various experimental and commercial rapid, solid-phase antigen-capture ELISAs (AC-ELISAs) are an effective alternative (Swayne *et al.*, 2013). Most AC-ELISAs have been licensed and marketed to detect human influenza A virus in clinical specimens. Some have demonstrated effectiveness for detection of influenza A, but many of these commercial tests have had low sensitivity (Woolcock & Cardona, 2005). Those validated for veterinary use are preferred.

Any HA activity of sterile fluids harvested from the inoculated eggs is most likely to be caused by an influenza A virus or an avian paramyxovirus, but a few strains of avian reovirus, as well as nonsterile fluid containing HA of bacterial origin can cause the agglutination of RBCs. There are currently 12 recognised serotypes of avian paramyxoviruses (Miller *et al.*, 2010). Most laboratories will have antiserum specific to Newcastle disease virus (avian paramyxovirus type 1), and in view of its widespread occurrence and almost universal use as a live vaccine in poultry, it is best to evaluate its presence by haemagglutination inhibition (HI) tests (see Chapter 2.3.14 *Newcastle disease*).

Alternatively, the presence of influenza virus can be confirmed by the use of RT-PCR or real-time RT-PCR using nucleoprotein-specific or matrix-specific conserved primers (Altmüller *et al.*, 1991; Spackman *et al.*, 2002). Also, the presence of subtype H5 or H7 influenza virus can be confirmed by using H5- or H7-specific primers (Monne *et al.*, 2008; Slomka *et al.*, 2007; Spackman *et al.*, 2002).

Antigenic subtyping can be accomplished by monospecific antisera prepared against purified or recombinant H and N subtype-specific proteins, used in HI and NI tests, or polyclonal antisera raised against a battery of intact influenza viruses and used in HI and NI tests. Genotyping can be accomplished using H and N subtype specific primers in RT-PCR and real-time RT-PCR tests; or using sequence analysis of H and N genes. Subtype identification by these techniques is beyond the scope of most diagnostic laboratories not specialising in influenza viruses. Assistance is available from the OIE Reference Laboratories (see Table given in Part 4 of this *Terrestrial Manual*).

1.1.1. Assessment of pathogenicity

The term HPAI relates to the assessment of pathogenicity in chickens and implies the involvement of highly pathogenic strains of virus. It is used to describe a disease of fully susceptible chickens with clinical signs that may include one or more of the following: ocular and nasal discharges, coughing, sneezing and dyspnoea, swelling of the sinuses and/or head, listlessness, reduced vocalisation, marked reduction in feed and water intake, cyanosis of the unfeathered skin, wattles and comb, incoordination, nervous signs and diarrhoea. In laying birds, additional clinical features include a marked drop in egg production usually accompanied by an increase in numbers of poor quality eggs. Typically, high morbidity is accompanied by high and rapidly escalating unexplained mortality. However, none of these signs can be considered pathognomonic and high mortality may occur in their absence. In addition, low pathogenicity influenza A viruses that normally cause only mild or no clinical disease, may cause a much more severe disease if exacerbating infections or adverse environmental factors are present and, in certain circumstances, the spectrum of clinical signs may mimic HPAI.

The historical term 'fowl plague' has been abandoned in favour of the more accurate term HPAI. Because all naturally occurring HPAI viruses to date have been H5 and H7 subtypes and genomic studies have determined HPAI viruses arise by mutation of H5/H7 LPAI viruses, all H5/H7 LPAI have been recognised as potentially pathogenic. Pathogenicity shifts have been associated with changes to the proteolytic cleavage site of the haemagglutinin including: 1) substitutions of non-basic with basic amino acids (arginine or lysine); 2) insertions of multiple basic amino acids from codons duplicated from the haemagglutinin cleavage site; 3) short inserts of basic and non-basic amino acids from unknown source; 4) recombination with inserts from other gene segments that lengthen the proteolytic cleavage site; and 5) loss of the shielding glycosylation site at residue 13 in combination with multiple basic amino acids at the cleavage site. Amino acid sequencing of the cleavage sites of H5 and H7 subtype influenza isolates of low pathogenicity for birds should identify viruses that have the capacity, following simple mutation, to become highly pathogenic for poultry.

The following criteria have been adopted by the OIE for determining pathogenicity of an influenza A virus:

- a) One of the two following methods to determine pathogenicity in chickens is used. A high pathogenicity influenza A virus is:
 - i) any influenza A virus that is lethal² for six, seven or eight of eight 4- to 8-week-old susceptible chickens within 10 days following intravenous inoculation with 0.2 ml of a 1/10 dilution of a bacteria-free, infective allantoic fluid

or

 - ii) any influenza A virus that has an intravenous pathogenicity index (IVPI) greater than 1.2. The following is the IVPI procedure:
 - Fresh infective allantoic fluid with a HA titre >1/16 (>24 or >log₂ 4 when expressed as the reciprocal) is diluted 1/10 in sterile isotonic saline.
 - 0.1 ml of the diluted virus is injected intravenously into each of ten 6-week-old SAN susceptible chickens; if possible, SPF chickens should be used.
 - Birds are examined at 24-hour intervals for 10 days. At each observation, each bird is scored 0 if normal, 1 if sick, 2 if severely sick, 3 if dead. (The judgement of sick and severely sick birds is a subjective clinical assessment. Normally, 'sick' birds would show one of the following signs and 'severely sick' more than one of the following signs: respiratory involvement, depression, diarrhoea, cyanosis of the exposed skin or wattles, oedema of the face and/or head, nervous signs. Dead individuals must be scored as 3 at each of the remaining daily observations after death³.)
 - The IVPI is the mean score per bird per observation over the 10-day period. An index of 3.00 means that all birds died within 24 hours, and an index of 0.00 means that no bird showed any clinical sign during the 10-day observation period.
- b) For all H5 and H7 viruses of low pathogenicity in chickens, the amino acid sequence of the connecting peptide of the haemagglutinin must be determined. If the sequence is similar to that observed for other HPAI isolates, the isolate being tested will be considered to be HPAI (see the table that lists all the reported haemagglutinin proteolytic cleavage site of HA0 protein for H5 and H7 LPAI and HPAI viruses based on deduced amino acid sequence, which can be found on the OFFLU site at:
http://www.offlu.net/fileadmin/home/en/resource-centre/pdf/Influenza_A_Cleavage_Sites.pdf.

The OIE has the following classification system to identify influenza A viruses for which disease reporting and control measures should be taken:

- a) All influenza A isolates from poultry that meet the above criteria are designated as HPAI and are notifiable.

² When birds are too sick to eat or drink, they should be killed humanely.

³ When birds are too sick to eat or drink, they should be killed humanely and scored as dead at the next observation.

- b) H5 and H7 isolates from poultry that are not highly pathogenic for chickens and do not have an HA0 cleavage site amino acid sequence similar to any of those that have been observed in HPAI viruses are designated as H5/H7 LPAI and are notifiable.
- c) For the purposes of the *Terrestrial Code*, HPAI and H5/H7 LPAI in poultry are termed “avian influenza” and are notifiable. Non-H5/H7 influenza A (i.e. H1–4, H6 and H8–16) are not “avian influenza” and are not notifiable.
- d) Influenza A viruses of high pathogenicity in birds other than poultry, including wild birds are notifiable.

A variety of strategies and techniques have been used successfully to sequence the nucleotides at that portion of the HA gene coding for the cleavage site region of the haemagglutinin of H5 and H7 subtypes of avian influenza virus, enabling the amino acids there to be deduced. This can be done by extraction the specimen and direct sequencing of the hemagglutinin proteolytic cleavage site, or first, cloning the hemagglutinin and then sequencing the cDNA. Various stages in the procedure can be facilitated using commercially available kits and automatic sequencers.

Determination of the cleavage site by sequencing or other methods has become the method of choice for initial assessment of the pathogenicity of these viruses and has been incorporated into agreed definitions. This has reduced the number of *in-vivo* tests, although at present the inoculation of birds is still required to confirm a negative result as the possibility of virus populations containing mixtures of viruses of high and low pathogenicity cannot be ruled out.

Although all the truly HPAI viruses isolated to date have been of H5 or H7 subtypes, at least two isolates, both of H10 subtype (H10N4 and H10N5), have been reported that would have fulfilled both the OIE and EU *in-vivo* definitions for HPAI viruses (Wood *et al.*, 1996) as they killed 7/10 and 8/10 chickens with IVPI values >1.2 when the birds were inoculated intravenously. However, these viruses did not induce death or signs of disease when inoculated intranasally and did not have a haemagglutinin cleavage site sequence compatible with HPAI virus. Similarly, other intravenously inoculated influenza A viruses are nephrotropic and birds that die have high titres of virus in their kidneys indicating a renal pathogenic mechanism (Slemons & Swayne, 1990), but such laboratory-induced pathobiology does not equal to the multi-organ infection and systemic disease caused by HPAI viruses. Conversely, four viruses have been described that have HA0 cleavage sites containing multiple basic amino acids, but which show low pathogenicity (IVPI <1.2) when inoculated intravenously into 6-week-old chickens (Londt *et al.*, 2007). Other anomalies are the Chile 2002 (Suarez *et al.*, 2004) and the Canada 2004 (Pasick *et al.*, 2005) H7N3 HPAI viruses, which show distinct and unusual cleavage site amino acid sequences of PEKPKTCSPLSRETR*GLF and PENPKQAYRKRMT*GLF, respectively. These viruses appear to have arisen as a result of a recombination between the HA, nucleoprotein and matrix genes, respectively, resulting in an insertion at the HA0 cleavage site of 11 amino acids for the Chile virus and 7 amino acids for the Canadian virus. Both are extremely pathogenic when inoculated into 6-week-old chickens intravenously.

A table is available on the OFFLU web site that lists all the reported haemagglutinin proteolytic cleavage site of HA0 protein for H5 and H7 LPAI and HPAI viruses based on deduced amino acid sequence. This table will be updated as new virus are characterised; it can be found on the OFFLU site at: http://www.offlu.net/fileadmin/home/en/resource-centre/pdf/Influenza_A_Cleavage_Sites.pdf

1.2. Antigen capture and molecular techniques

At present, the conventional virus isolation and characterisation techniques for the diagnosis of influenza A remain the methods of choice, for at least the initial diagnosis of influenza A infections. However, conventional methods tend to be costly, labour intensive and slow. There have been enormous developments and improvements in molecular and other diagnostic techniques, many of which have been applied to the diagnosis of influenza A infections.

1.2.1. Antigen detection

There are several commercially available AC-ELISA kits that can detect the presence of influenza A viruses in poultry (Swayne *et al.*, 2013; Woolcock & Cardona, 2005). Most of the kits are enzyme immunoassays or are based on immunochromatography (lateral flow devices) and use a monoclonal antibody against the nucleoprotein; they should be able to detect any influenza A virus. The main advantage of these tests is that they can demonstrate the presence of influenza A within 15 minutes. The disadvantages are that they may lack sensitivity, they may not have been validated for different species of birds, subtype identification is not achieved and the kits are expensive. The tests should only be interpreted on a flock basis and not as an individual bird test. Oropharyngeal or tracheal samples from clinically affected or dead birds

provide the best sensitivity. Nevertheless, the lack of sensitivity is a major drawback to the use of available antigen detection tests. Chua *et al.* (Chua *et al.*, 2007) evaluated five detection tests and showed overall sensitivities from 36.3% to 51.4%; these authors pointed out that in terms of sensitivity using cloacal and tracheal swabs, the tests performed less well with samples from waterfowl or wild birds than they did with samples from chickens. Woolcock & Cardonna (Woolcock & Cardonna, 2005) examined five commercial tests licensed for human clinical use and found a wide variation in the ability to detect influenza A virus in poultry specimens with minimal detection limits of $10^{4.7}$ EID₅₀ (50% egg-infective dose) of virus per ml with the best test, and a minimum of $10^{5.7}$ EID₅₀ per ml for the remaining tests. Because of low sensitivity, antigen detection is mainly used for field screening of high mortality clinical cases for influenza A followed by confirmation of results using a more sensitive laboratory based test.

1.2.2. Direct RNA detection

As demonstrated by the current definitions of HPAI, molecular techniques have been used in diagnosis for some time now. Furthermore, there have recently been developments towards their application to the detection and characterisation of influenza A viruses directly from clinical specimens of infected birds. It is imperative that when using highly sensitive molecular detection methods that allow rapid direct detection of viral RNA for confirmatory laboratory diagnosis of influenza A infections, stringent protocols are in place to prevent the risk of cross-contamination between clinical samples. In addition, RNA detection test methodologies should be validated to the OIE standard (see Chapter 1.1.6 *Principles and methods of validation of diagnostic assays for infectious diseases*) using clinical material to demonstrate the tests as being 'fit for purpose' for application in a field diagnostic setting, which may include the use of internal test standards. The control reactions enable greater confidence in the integrity of the molecular reactions, clinical samples and results.

RT-PCR techniques on clinical specimens can, with the correctly defined primers, result in rapid detection and subtype identification (at least of H5 and H7), including a cDNA product that can be used for nucleotide sequencing (Suarez, 2007). This technique was used with success during the 2003 HPAI outbreaks in The Netherlands. Gall *et al.* (2008; 2009) have developed degenerate primers for detection and sequencing of short HA (cleavage site) and NA gene fragments which allow amplification across all HA (1–16) and NA (1–9) subtypes. However, the preferred molecular detection tests for influenza A virus is the real-time RT-PCR, a modification to the RT-PCR that reduces the time for both identification of virus subtype and sequencing. For example, Spackman *et al.* (2002) used a single-step real-time RT-PCR primer/fluorogenic hydrolysis probe system to allow detection of influenza A viruses and determination of subtype H5 or H7. The test performed well relative to virus isolation and offered a cheaper and much more rapid alternative, with diagnosis on clinical samples in less than 3 hours. In additional studies, the real-time RT-PCR was shown to have sensitivity and specificity equivalent to virus isolation based on field validation in the live poultry market control programme of New York and New Jersey during the winter of 2002, and the H7N2 LPAI outbreak and eradication programme in Virginia during 2002 (Elvinger *et al.*, 2007; Spackman *et al.*, 2003). The test provides high sensitivity and specificity similar to virus isolation from tracheal and oropharyngeal swabs of chickens and turkeys, but may lack sensitivity for detection of influenza A virus in faecal swabs, faeces and tissues in some bird species, because of the presence of PCR inhibitors resulting in false-negative results (Das *et al.*, 2006). Incorporation of a positive internal control into the test will verify a proper test run. In addition, improved RNA extraction methods have been developed to eliminate most PCR inhibitors from test samples.

Real-time RT-PCR, usually based around the hydrolysis probe or 'TaqMan®' method for generation of the target-specific fluorescence signal, has become the method of choice in many laboratories for at least partial diagnosis directly from clinical specimens. The method offers rapid results, with sensitivity and specificity comparable to virus isolation. These are ideal qualities for influenza A outbreak management, where the period of time in which an unequivocal diagnosis can be obtained is crucial for decision making by the relevant Veterinary Authority. In addition, real-time RT-PCR systems can be designed to operate in a 96-well format and combined with high-throughput robotic RNA extraction from specimens (Agüero *et al.*, 2007).

The approach to diagnosis using real-time RT-PCR adopted in most laboratories has been based on initial generic detection of influenza A virus in clinical specimens, primarily by initially targeting the matrix (M) gene, which is highly conserved for all influenza A viruses, followed by specific real-time RT-PCR testing for H5 and H7 subtype viruses. For subtype identification, primers used in TaqMan real-time RT-PCRs are targeted at the HA2 region, as this is relatively well conserved within the haemagglutinin genes of the H5 and H7 subtypes (Spackman *et al.*,

2008; Spackman & Suarez, 2008). It has therefore served as the target region for these subtypes. Spackman *et al.* (2002) demonstrated specific detection of these subtypes, but cautioned that their H5 and H7 primer/probe sequences had been designed for the detection of North American H5 and H7 isolates and might not be suitable for all H5 and H7 isolates. This proved to be the case. Slomka *et al.* (Slomka *et al.*, 2007) described modification of the H5 oligonucleotide sequences used by Spackman *et al.* (2002) to enable the detection of the Asian lineage H5N1 subtype and other Eurasian H5 subtypes that have been isolated within the past decade in both poultry and wild birds. Validated real-time RT-PCR protocols for the simultaneous detection and typing of H5, H7 and H9 RNA have been developed (Monne *et al.*, 2008). These validated Eurasian real-time RT-PCR have proven valuable in the investigation of many H5N1 HPAI clinical specimens and other subtypes submitted to International Reference Laboratories from Europe, Africa and Asia since autumn 2005 (Monne *et al.*, 2008; Slomka *et al.*, 2007). Each set of primers and probes needs to be validated against a diverse set of viruses to make the test applicable to in diverse avian species, and in viruses from broad geographic areas and time periods.

One of the problems with rapidly emerging new tests is that methods and protocols may be developed and reported without the test being properly validated. This has been addressed for some of the real-time RT-PCR protocols (Slomka *et al.*, 2007b; Suarez *et al.*, 2007). In the European Union, National Reference Laboratories have collaborated to define and validate protocols that can be recommended for use within the European Union (Monne *et al.*, 2008; Slomka *et al.* 2007b).

Real-time RT-PCR protocols have been described that amplify regions across the cleavage site of the HA0 gene. This may result in useful tests for specific viruses. For example, Hoffman *et al.* (2007) have described a real-time RT-PCR test specific to the Asian HPAI H5N1 Qinghai-like clade 2.2 viruses that represents a rapid means of determining the pathotype for this subgroup of H5N1 HPAI viruses without sequencing. Fereidouni *et al.* (2008) have developed a restriction fragment polymorphism-based assay that enables the pathotyping of influenza A of subtype H5 independent of sequencing or animal experiments after RT-PCR and restriction enzyme digest of the amplicate.

Modifications to the straightforward RT-PCR method of detection of viral RNA have been designed to reduce the effect of inhibitory substances in the sample taken, the possibility of contaminating nucleic acids and the time taken to produce a result. For example, nucleic acid sequence-based amplification (NASBA) with electrochemiluminescent detection (NASBA/ECL) is a continuous isothermal reaction in which specialised thermocycling equipment is not required. NASBA assays have been developed for the detection of avian influenza virus subtypes H7 and H5 in clinical samples within 6 hours (Ko *et al.*, 2004). The loop-mediated isothermal amplification (LAMP) system for H5 detection appeared to show high sensitivity and reliable specificity (Imai *et al.*, 2006), but may have limited application because of susceptibility to viral mutations affecting the target regions, reducing virus detection (Postel *et al.*, 2010).

It seems highly likely that within a very short time molecular-based and improved antigen-based technologies will have developed sufficiently to allow rapid 'flock-side' tests for the detection of the presence of influenza A virus specific subtypes and pathogenicity markers. The extent to which such tests are employed will depend very much on the agreement on and adoption of definitions of what constitutes statutory infections for control and trade purposes. Currently, real-time RT-PCR is the preferred method for virus surveillance because the test provides rapid, sensitive diagnostics for influenza A, H5 and H7, and is amenable for high throughput.

2. Serological tests

2.1. Enzyme-linked immunoassay (ELISA)

Commercial ELISA kits that detect antibodies against the nucleocapsid protein are available. Kits with an indirect and competitive/blocking format have been developed and validated, and are now being used to detect influenza A virus-specific antibodies. Several avian influenza competitive ELISA (AIV C-ELISA) or blocking ELISA (AIV B-ELISA) have been developed and validated as a more sensitive alternative to the AGID test for the detection of influenza A group reactive antibodies in sera from chickens and other bird species (SCAHLs, 2009). This AIV ELISA platform, as either a "competitive" or "blocking" format, detects antibodies to influenza A viruses by allowing these antibodies to compete for antigen binding sites with a monoclonal antibody against an epitope on the nucleoprotein that is conserved in all influenza A viruses.

The kits should be validated for the specific species of interest and for the specific purpose(s) for which they are to be used. Several different test and antigen preparation methods are used. Such tests have usually been evaluated and validated by the manufacturer, and it is therefore important that the instructions specified for their use be followed carefully. Please see the OIE Register for kits certified by the OIE (<http://www.oie.int/en/our-scientific-expertise/registration-of-diagnostic-kits/background-information/>). ELISA kits are of moderate cost and are amenable to high throughput screening for influenza A virus infections, but all positive results must be followed by HI test for subtyping to H5 and H7. Some subtype-specific ELISA kits are becoming available, e.g. for antibodies to H5, H7 and N1.

2.2. Agar gel immunodiffusion

All influenza A viruses have antigenically similar nucleocapsid and antigenically similar matrix antigens. Owing to this fact AGID tests are able to detect the presence or absence of antibodies to any influenza A virus. Concentrated virus preparations, as described above, contain both matrix and nucleocapsid antigens; the matrix antigen diffuses more rapidly than the nucleocapsid antigen. AGID tests have been widely and routinely used to detect specific antibodies in chicken and turkey flocks as an indication of infection, but AGID tests are less reliable at detecting antibodies following infection with influenza A viruses in other avian species. These have generally employed nucleocapsid-enriched preparations made from the chorioallantoic membranes of embryonated chicken eggs (Beard, 1970) that have been infected at 10 days of age, homogenised, freeze–thawed three times, and centrifuged at 1000 *g*. The supernatant fluids are inactivated by the addition of 0.1% formalin or 1% betapropiolactone, recentrifuged and used as antigen. Not all avian species may produce precipitating antibodies following infection with influenza viruses, for example ducks. The AGID is a low cost serological screening test for detection of generic influenza A infections, but must be followed by HI tests for subtyping influenza A positives as to H5 and H7.

Tests are usually carried out using gels of 1% (w/v) agarose or purified type II agar and 8% (w/v) NaCl in 0.01 M phosphate buffer, pH 7.2, poured to a thickness of 2–3 mm in Petri dishes or on microscope slides, and incubated in a humidified chamber. Using a template and cutter, wells of approximately 5 mm in diameter are cut into the agar. A pattern of wells must place each suspect serum adjacent to a known positive serum and antigen. Each well should have reagent added to fill the well, corresponding to the top of the meniscus with the top of the gel, but do not over fill. Approximately 50 µl of each reagent should be required per well, but this depends on thickness of the gel, with thicker gels requiring an additional volume of reagent.

Wells should be examined for precipitin lines at 24 hours, and weak positive samples or samples for which specific lines have not formed should be incubated longer and examined again at 48 hours. The time to formation of visible precipitin line is dependent on the concentrations of the antibody and the antigen. The precipitin lines are best observed against a dark background that is illuminated from behind. A specific, positive result is recorded when the precipitin line between the known positive control wells is continuous with the line between the antigen and the test well. Crossed lines are interpreted to be caused by the test serum lacking identity with the antibodies in the positive control well.

2.3. Haemagglutination and haemagglutination inhibition tests

Variations in the procedures for HA and HI tests are practised in different laboratories. The following recommended examples apply to the use of V-bottomed microwell plastic plates in which the final volume for both types of test is 0.075 ml. The reagents required for these tests are isotonic PBS (0.01 M), pH 7.0–7.2, and red blood cells (RBCs) taken from a minimum of three SPF or SAN chickens and pooled into an equal volume of Alsever's solution. Cells should be washed three times in PBS before use as a 1% (packed cell v/v) suspension. Positive and negative control antigens and antisera should be run with each test, as appropriate.

2.3.1. Haemagglutination test

- i) Dispense 0.025 ml of PBS into each well of a plastic V-bottomed microtitre plate.
- ii) Place 0.025 ml of virus suspension (i.e. infective allantoic fluid) in the first well. For accurate determination of the HA content, this should be done from a close range of an initial series of dilutions, i.e. 1/3, 1/4, 1/5, 1/6, etc.
- iii) Make twofold dilutions of 0.025 ml volumes of the virus suspension across the plate.
- iv) Dispense a further 0.025 ml of PBS to each well.

- v) Dispense 0.025 ml of 1% (v/v) chicken RBCs to each well.
- vi) Mix by tapping the plate gently and then allow the RBCs to settle for about 40 minutes at room temperature, i.e. about 20°C, or for 60 minutes at 4°C, if ambient temperatures are high, by which time control RBCs should have formed a distinct button.
- vii) HA is determined by tilting the plate and observing the presence or absence of tear-shaped streaming of the RBCs. The titration should be read to the highest dilution giving complete HA (no streaming); this represents 1 HA unit (HAU) and can be calculated accurately from the initial range of dilutions.

2.3.2. Haemagglutination inhibition test

- i) Dispense 0.025 ml of PBS into each well of a plastic V-bottomed microtitre plate.
- ii) Place 0.025 ml of serum into the first well of the plate.
- iii) Make twofold dilutions of 0.025 ml volumes of the serum across the plate.
- iv) Add 4 HAU of virus/antigen in 0.025 ml to each well and leave for a minimum of 30 minutes at room temperature (i.e. about 20°C) or 60 minutes at 4°C.
- v) Add 0.025 ml of 1% (v/v) chicken RBCs to each well and mix gently, allow the RBCs to settle for about 40 minutes at room temperature, i.e. about 20°C, or for 60 minutes at 4°C if ambient temperatures are high, by which time control RBCs should have formed a distinct button.
- vi) The HI titre is the highest dilution of serum causing complete inhibition of 4 HAU of antigen. The agglutination is assessed by tilting the plates. Only those wells in which the RBCs stream at the same rate as the control wells (containing 0.025 ml RBCs and 0.05 ml PBS only) should be considered to show inhibition.
- vii) The validity of results should be assessed against a negative control serum, which should not give a titre $>1/4$ ($>2^2$ or $>\log_2 2$ when expressed as the reciprocal), and a positive control serum for which the titre should be within one dilution of the known titre.

The HI test is primarily used to determine if antibodies indicating influenza A virus infections are subtyped as H5 and H7. HI titres may be regarded as being positive if there is inhibition at a serum dilution of 1/16 (2^4 or $\log_2 4$ when expressed as the reciprocal) or more against 4 HAU of antigen. Some laboratories prefer to use 8 HAU in HI tests. While this is permissible, it affects the interpretation of results so that a positive titre is 1/8 (2^3 or $\log_2 3$) or more. The meaning of a minimum positive titre should not be misinterpreted; it does not imply, for example, that immunised birds with that titre will be protected against challenge or that birds with lower titres will be susceptible to challenge. Appropriate virus/antigen control, positive control serum and RBC control well should be included with each batch of HI tests.

Chicken sera rarely give nonspecific positive agglutination reactions in this test and any pretreatment of the sera is unnecessary. Sera from species other than chickens may sometimes cause agglutination of chicken RBCs resulting in nonspecific agglutination. Therefore, each serum should first be tested for this idiosyncrasy and, if present, it should be inhibited by adsorption of the serum with chicken RBCs. This is done by adding 0.025 ml of packed chicken RBCs to each 0.5 ml of antisera, mixing gently and leaving for at least 30 minutes; the RBCs are then pelleted by centrifugation at 800 **g** for 2–5 minutes and the adsorbed sera are decanted. Alternatively, RBCs of the avian species under investigation could be used. Nonspecific inhibition of agglutination can be caused by steric inhibition when the H antigen and serum in the HI test have the same N subtype. The steric inhibition reaction can result in RBC buttoning in the bottom of the plate or streaming at the same rate as the control. To prevent steric nonspecific inhibition the H antigen used to test unknown serum must be of a different N subtype than the unknown sera, or the H antigen use can be recombinant or purified H protein that lacks N protein. The HI test is based on antigenic binding between the H antigen and antisera and thus other factors may cause nonspecific binding of the H antigen and sera leading to a nonspecific inhibition reaction. At this time there are no documented cross reactions or nonspecific inhibition reactions between the different haemagglutinin subtypes of influenza A.

The neuraminidase-inhibition test has been used to identify the influenza A neuraminidase type of isolates as well as to characterise the antibody in infected birds. The procedure requires specialised expertise and reagents; consequently this testing is usually done in an OIE Reference Laboratory. The DIVA (differentiating infected from vaccinated animals) strategy used in Italy also relies on a serological test to detect specific anti-N antibodies; the test procedure has been described (Capua *et al.*, 2003).

C. REQUIREMENTS FOR VACCINES

1. Background

It is important that vaccination alone is not considered the solution to the control of HPAI or H5/H7 LPAI subtypes if eradication is the desired result. Without the application of monitoring systems, strict biosecurity and depopulation in the face of infection, there is the possibility that HPAI and H5/H7 LPAI viruses could become endemic in vaccinated poultry populations. Long-term circulation of the virus in a vaccinated population may result in both antigenic and genetic changes in the virus and this has been reported to have occurred in Mexico, China (People's Rep. of), Egypt, Indonesia and other countries (Grund *et al.*, 2011; Lee *et al.*, 2004; Smith *et al.*, 2006; Swayne & Kapczynski, 2008b). Currently used vaccines and the use of vaccination have been reviewed (Capua & Alexander, 2008; Swayne, 2003, 2004; 2012b; Swayne *et al.*, 2011).

In this chapter, conventional vaccines are limited to inactivated influenza A virus vaccines. These vaccines have been used against HPAI, H5/H7 LPAI or non-H5/H7 influenza A having been prepared from infective allantoic fluid inactivated by beta-propiolactone or formalin and emulsified with mineral oil. Live conventional influenza vaccines against any subtype are not recommended.

The existence of a large number of virus subtypes, together with the known variation of different strains within a subtype, pose serious problems when selecting strains to produce inactivated influenza A vaccines. In addition, some isolates do not grow to a sufficiently high titre to produce adequately potent vaccines without costly pre-concentration. While some vaccination strategies use autogenous vaccines, i.e. vaccines prepared from isolates specifically involved in an epizootic, others rely on vaccines prepared from viruses possessing the same haemagglutinin subtype as the field virus and capable of yielding high concentrations of antigen.

Since the 1970s in the USA, inactivated influenza A vaccines have been used primarily in turkeys against H5/H7 LPAI and non-H5/H7 influenza A viruses. These viruses may cause severe clinical signs, especially in exacerbating circumstances. Significant quantities of this vaccine have been used (Swayne *et al.*, 2013). In recent years in the USA, most of the inactivated influenza A vaccine has been used in breeder turkeys to protect against H1 and H3 swine influenza viruses. Vaccination against H9N2 influenza A virus has been used extensively in Asia and the Middle East (Swayne & Kapczynski, 2008a). Vaccination against HPAI of H5N2 subtype was used in Mexico following outbreaks in 1994–1995 (Villareal, 2007), and against H7N3 subtype in Pakistan (Naeem, 1998) following outbreaks in 1995. In Mexico, the HPAI virus was eradicated, but LPAI virus of H5N2 has continued to circulate, while in Pakistan, HPAI viruses genetically close to the original HPAI virus were still being isolated in 2004. Following the outbreaks of HPAI caused by H5N1 virus in Hong Kong in 2002 (Sims, 2003), a vaccination policy was adopted using H5N2 vaccine and subsequently replaced with H5N1 vaccine. Beginning in 2004, the widespread outbreaks of H5N1 HPAI in several countries of South-East Asia and Africa resulted in emergency and prophylactic vaccination being applied in China (the People's Rep. of), Indonesia, Vietnam and Egypt. Inactivated H7N7 influenza A vaccine was used in Korea (Dem. Rep. of) during 2005 to control a HPAI outbreak. Similarly, preventive vaccination against H5N1 HPAI has been permitted for outdoor poultry and zoo birds in several European Union countries in recent years. Italy has extensively used the tool of serological (heterologous neuraminidase) DIVA with vaccination to control recurrent epidemics of H7 LPAI. A bivalent H5/H7 prophylactic vaccination programme was also developed as a result of an evolving epidemiological situation (Capua & Marangon, 2008).

Live recombinant virus-vectored vaccines with H5 influenza A virus haemagglutinin gene inserts have been licensed and used in a few countries since 1997, mostly in chickens, and include recombinant fowl poxvirus, recombinant Newcastle disease virus and recombinant herpesvirus turkey vaccines. A recombinant duck enteritis virus in domestic ducks is being tested for potential licensure and use in China (People's Rep. of) (Liu *et al.*, 2011).

1.1. Rationale and intended use of the product

Experimental work has shown, for HPAI and H5/H7 LPAI, that properly administered vaccines protect against clinical signs and mortality, reduces virus shedding and increases resistance to infection, protects from diverse field viruses within the same haemagglutinin subtype, protects from low and high challenge exposure, and reduces excretion and thus contact transmission of challenge virus (Capua *et al.*, 2004; Swayne, 2003; Swayne & Suarez, 2000). However, the virus is still able to infect and replicate in clinically healthy vaccinated SPF birds when given in high challenge doses. Most of the work evaluating vaccines has been done in chickens and turkeys and some care must be taken in extrapolating the results obtained to other species. For example, in an experimental system using H7N7 HPAI as a challenge virus it was shown for chickens and ringed teal ducks, *Callonetta leucophrys*, that single vaccination sufficiently reduced excretion and increased the infective dose required and the transmission between birds was dramatically reduced. However, for golden pheasants, *Chrysolophus pictus*, even though a single vaccination provided clinical protection, there was no effect on the excretion of challenge virus and no influence on reducing virus transmission (Van

der Goot *et al.*, 2007). In some countries, vaccines designed to contain or prevent HPAI and H5/H7 LPAI are specifically banned or discouraged by government agencies because it has been considered that they may interfere with stamping-out control policies. However, most HPAI and H5/H7 LPAI control regulations reserve the right to use vaccines in emergencies.

2. Outline of production and minimum requirements for conventional vaccines

The information below is based primarily on the experiences in the USA and the guidance and policy for licensing influenza A vaccines in that country (United States Department of Agriculture, 1995 [updated 2006]). The basic principles for producing vaccines, particularly inactivated vaccines, are common to several viruses e.g. Newcastle disease (chapter 2.3.14).

Guidelines for the production of veterinary vaccines are given in Chapter 1.1.8 *Principles of veterinary vaccine production*. The guidelines given here and in chapter 1.1.8 are intended to be general in nature and may be supplemented by national and regional requirements.

The vaccine production facility should operate under the appropriate biosecurity procedures and practices. If HPAI virus is to be used in challenge studies, the facility used for such studies should meet the requirements for Containment Group 4 pathogens as outlined in chapter 1.1.4.

2.1. Characteristics of the seed

2.1.1. Biological characteristics

For any subtype, only well characterised influenza A virus of proven low pathogenicity, preferably obtained from an international or national repository, should be used to establish a master seed for inactivated vaccines. HPAI viruses should not be used as seed virus for vaccine. For HPAI, reverse genetic produced vaccine seed strains based on haemagglutinin gene of the HPAI virus are preferred, but should have the cleavage site sequence altered to that of a H5/H7 LPAI virus.

A master seed is established from which a working seed is obtained. The master seed and working seed are produced in SPF or SAN embryonated eggs. The establishment of a master culture may only involve producing a large volume of infective allantoic fluid (minimum 100 ml), which can be stored as lyophilised aliquots (0.5 ml).

2.1.2. Quality criteria (sterility, purity, freedom from extraneous agents)

The established master seed should be controlled/examined for sterility, safety, potency and absence of specified extraneous agents.

2.2. Method of manufacture

2.2.1. Procedure

For vaccine production, a working seed, from which batches of vaccine are produced, is first established in SPF or SAN embryonated eggs by expansion of an aliquot of master seed to a sufficient volume to allow vaccine production for 12–18 months. It is best to store the working seed in liquid form at below -60°C as lyophilised virus does not always multiply to high titre on subsequent first passage.

The routine procedure is to dilute the working seed in sterile isotonic buffer (e.g. PBS, pH 7.2), so that about 10^3 – 10^4 EID₅₀ in 0.1 ml are inoculated into each allantoic cavity of 9- to 11-day-old embryonated SPF or SAN chicken eggs. These are then incubated at 37°C . Eggs containing embryos that die within 24 hours should be discarded. The incubation time will depend on the virus strain being used and will be predetermined to ensure maximum yield with the minimum number of embryo deaths.

The infected eggs should be chilled at 4°C before being harvested. The tops of the eggs are removed and the allantoic fluids collected by suction. The inclusion of any yolk material and albumin should be avoided. All fluids should be stored immediately at 4°C and tested for bacterial contamination.

In the manufacture of inactivated vaccines, the harvested allantoic fluid is treated with either formaldehyde (a typical final concentration is 1/1000, i.e. 0.1% formalin) or beta-propiolactone (BPL) (a typical final concentration is 1/1000–1/4000, i.e. 0.1–0.025% of 99% pure BPL). The

time required must be sufficient to ensure freedom from live virus. Most inactivated vaccines are formulated with non-concentrated inactivated allantoic fluid (active ingredient). However, active ingredients may be concentrated for easier storage of antigen. The active ingredient is usually emulsified with mineral or vegetable oil. The exact formulations are generally commercial secrets.

2.2.2. Requirements for substrates and media

The inactivated influenza A vaccines prepared from conventional virus are produced in 9- to 11-day-old embryonated SPF or SAN chicken eggs. The method of production is basically the same as for propagating the virus aseptically; all procedures are performed under sterile conditions.

2.2.3. In-process controls

For inactivated vaccines, completion of the inactivation process should be tested in embryonated eggs, taking at least 10 aliquots of 0.2 ml from each batch and passaging each aliquot at least twice through SPF or SAN embryos. Viral infectivity must not remain.

2.2.4. Final product batch tests

Most countries have published specifications for the control of production and testing of vaccines, which include the definition of the obligatory tests on vaccines during and after manufacture.

i) Sterility and purity

Tests of biological materials for sterility and freedom from contamination may be found in chapter 1.1.9.

ii) Safety

For inactivated vaccines, a double dose is administered by the recommended route to ten 3-week-old birds, and these are observed for 2 weeks for absence of clinical signs of disease or local lesions.

iii) Batch potency

Potency of influenza A vaccine is generally evaluated by testing the ability of the vaccine to induce a significant HI titre in SPF or SAN birds. Conventional potency testing involving the use of three diluted doses and challenge with HPAI virus (e.g. chapter 2.3.14) may also be used for vaccines prepared to give protection against HPAI or H5/H7 LPAI subtypes. For inactivated vaccines to other subtypes, where HPAI viruses are not available, potency tests may rely on the measurement of immune response or challenge and assessment of morbidity and quantitative reduction in challenge virus replication in respiratory (oropharyngeal or tracheal) and intestinal (cloaca) tracts. Assessment of haemagglutinin antigen content (Wood *et al.*, 1985) could allow for *in-vitro* extrapolation to potency for subsequent vaccine batches.

iv) Preservatives

A preservative may be used for vaccine in multidose containers.

2.3. Requirements for authorisation

2.3.1. Safety requirements

i) Target and non-target animal safety

Most inactivated influenza A vaccines are licensed for use in chickens and turkeys. Field trials in the target species should be conducted to determine tolerance and safety of the vaccine at full dose. Recently the use of inactivated influenza A vaccines has been expanded to ducks, geese, other poultry and zoo birds. Any extra-label use of the vaccines should be done cautiously and under the supervision of a veterinarian experienced in disease control through vaccination in the test species. Care must be taken to avoid self-injection with oil emulsion vaccines.

ii) Reversion-to-virulence for attenuated/live vaccines

Only inactivated influenza A virus vaccines are recommended. Live conventional influenza vaccines against any subtype are not recommended because of the risk for reassortment of gene segments of vaccine virus with field virus, potentially creating more pathogenic field viruses.

iii) Environmental consideration

None

2.3.2. Efficacy requirements

i) For animal production

For licensing purposes, influenza A vaccines should pass an efficacy challenge test using a statistically relevant number of SPF or SAN chickens per group. The challenge should occur at a minimum of three weeks post-vaccination, using a challenge HPAI virus dose that causes 90% or greater mortality in the sham population. A standardised challenge dose of 10^6 mean chicken embryo infectious doses is most widely used. Protection from mortality in the vaccine group should be a minimum of 80%. For H5/H7 LPAI, mortality is not a feature of challenge models, therefore a statistically significant reduction in virus shedding titre and/or the number of birds shedding virus from oropharynx or cloaca should be observed between sham and test vaccine groups.

In establishing minimum antigen requirements, 50 PD₅₀ or 3 µg of haemagglutinin per dose have been suggested (Swayne & Kapczynski, 2008a). Minimum HI serological titres in field birds should be 1/32 to protect from mortality or greater than 1/128 to provide reduction in challenge virus replication and shedding.

ii) For control and eradication

Efficacy should be the same as for animal production.

2.3.3. Stability

When stored under the recommended conditions, the final vaccine product should maintain its potency for at least 1 year. Inactivated vaccines must not be frozen.

3. Vaccines based on biotechnology

3.1. Vaccines available and their advantages

Recombinant vaccines for influenza A viruses have been produced by inserting the gene coding for the influenza A virus haemagglutinin into a non-influenza live virus vector and using this recombinant virus to immunise poultry against influenza A (Swayne, 2004). Recombinant live vector vaccines have several advantages: 1) they are live vaccines able to induce mucosal, humoral and cellular immunity; 2) they can be administered to young birds and induce an early protection, e.g. the fowl poxvirus can be administered at 1 day of age, is compatible with the Marek's disease vaccine, and provides significant protection 1 week later; 3) they enable differentiation between infected and vaccinated birds, as, for example, they do not induce the production of antibodies against the nucleoprotein or matrix antigens that are common to all influenza A viruses. Therefore, only field-infected birds will exhibit antibodies in the AGID test or ELISAs directed towards the detection of influenza group A (nucleoprotein and/or matrix) antibodies. However, these vaccines have limitations in that they will replicate poorly and induce only partial protective immunity in birds that have had field exposure to or vaccination with the vector virus, i.e. fowl poxvirus or Newcastle disease viruses for currently available recombinant vaccines (Swayne & Kapczynski, 2008a; b). If used in day-old or young birds, the effect of maternal antibodies to the vector virus on vaccine efficacy may vary with the vector type. In the case of fowl poxvirus recombinant vaccine, it has been reported that effective immunisation was achieved when given to 1-day-old chicks with varying levels of maternal immunity (Arriola *et al.*, 1999). However, when very high levels of maternal antibodies are anticipated because of previous infection or vaccination, the efficacy of the fowlpox vector vaccine in such day-old chicks should be confirmed and may require a prime-boost application of recombinant vaccine followed in 2–3 weeks by inactivated influenza A vaccine boost. In addition, because the vectors are live viruses that may have a restricted host range (for example infectious laryngotracheitis virus does not replicate in turkeys), the use of these vaccines must be restricted to species in which efficacy has been demonstrated.

The use of recombinant vaccines is restricted to countries in which they are licensed and legally available. The recombinant fowlpox-influenza A-H5 vaccine has been licensed in El Salvador, Guatemala, Mexico, China (the People's Rep. of) and the USA (Swayne & Kapczynski, 2008a). Recombinant fowlpox virus vaccines containing H5 HA have been prepared and evaluated in field trials, but the only field experience with this vaccine has been in Mexico, El Salvador, Guatemala and China (the People's Rep. of) where it has been used in the vaccination campaign against the H5N2 LPAI and H5N1 HPAI viruses.

Newcastle disease virus can also be used as a vector for expressing influenza HA haemagglutinin genes. A recombinant Newcastle disease vaccine virus expressing a H5 HA gene was shown to protect chickens against challenge with both virulent Newcastle disease virus and a HPAI H5N2 virus (Veits *et al.*, 2006). A similar recombinant virus based on Newcastle disease virus vaccine strain La Sota and expressing the Asian lineage H5 HA gene was produced in China (the People's Rep. of) (Ge *et al.*, 2007) and reported to be efficacious in protection studies with either virus. This latter virus has been licensed in China (the People's Rep. of) and used widely. Recombinant Newcastle disease virus (rNDV) vaccines are effective in poultry lacking immunity to the Newcastle disease virus vector, but rNDV vaccines are largely ineffective as single dose primary vaccine in poultry with maternal immunity or well-immunised against Newcastle disease. rNDV vaccines are effective if used as a priming vaccine followed by a boost with an inactivated influenza A vaccine.

Recently, two additional virus vectored vaccines with H5 influenza A gene inserts have been developed: 1) a recombinant herpesvirus turkey (rHVT), and 2) a recombinant duck enteritis virus (rDEV) (Swayne & Spackman, 2013). The former has been licensed in Egypt and USA, while the latter is undergoing the licensing process in China (People's Rep. of). Both have demonstrated efficacy in the laboratory by protecting against H5N1 HPAI challenge in chickens and domestic ducks, respectively (Liu *et al.*, 2011; Rauw *et al.*, 2011). However, field reports of protection with vectored and conventional influenza A vaccines suggest that protection by single dose of the vectored vaccines is not feasible, with field protection requiring priming with vectored vaccine followed by a booster with inactivated influenza A vaccine or the vectored vaccine (Swayne, 2012a).

In addition to these licensed vaccines, various experimental haemagglutinin-based H5 and H7 influenza A vaccines have been described using *in-vivo* or *in-vitro* expression systems including recombinant adenoviruses, salmonella, baculovirus, vaccinia, avian leucosis virus, alphavirus and infectious laryngotracheitis virus (Swayne & Kapczynski, 2008a). DNA encoding H5 haemagglutinin has been evaluated as a potential vaccine in poultry (Rao *et al.*, 2008).

3.2. Special requirements for biotechnological vaccines, if any

Live recombinant vectored vaccines with H5 and H7 influenza A gene inserts should have an environmental impact assessment completed to determine the risk of the vaccine to be virulent in non-target avian species and will not increase in virulence in the target avian species.

4. Surveillance methods for detecting infection in vaccinated flocks and vaccinated birds

A strategy that allows differentiation of infected from vaccinated animals (DIVA), has been put forward as a possible solution to the eventual eradication of HPAI and H5/H7 LPAI without involving mass culling of birds and the resulting economic damage, especially in developing countries (FAO, 2004). This strategy has the benefits of vaccination (less virus in the environment), but the ability to identify infected flocks would still allow the implementation of additional control measures, including stamping out. DIVA strategies use one of two broad detection schemes within the vaccinated population: 1) detection of influenza A virus ('virus DIVA'), or 2) detection of antibodies against influenza A field virus infection ('serological DIVA'). At the flock level, a simple method consists of regularly monitoring sentinel birds left unvaccinated in each vaccinated flock, but this approach does have some management problems, particularly with regards to identifying the sentinels in large flocks. As an alternative or adjunct system, testing for field exposure may be performed on the vaccinated birds either by detection of field virus or antibodies against the virus. To detect the field virus, oropharyngeal or cloacal swabs from baseline daily mortality or sick birds can be tested, individually or as pools, by molecular methods, such as real-time RT-PCR or AC-ELISA of the vaccinated populations (Swayne & Kapczynski, 2008a).

To use serological DIVA schemes, vaccination systems that enable the detection of field exposure in vaccinated populations should be used. Several systems have been developed in recent years. These include the use of a vaccine containing a virus of the same haemagglutinin (H) subtype but a different neuraminidase (N) from the field virus. Antibodies to the N of the field virus act as natural markers of infection. This system has been used in Italy following the re-emergence of a H7N1 LPAI virus in 2000. In order to supplement direct control measures, a 'DIVA' strategy was implemented using a vaccine containing H7N3 to combat a H7N1 field infection. Vaccinated and field exposed birds were differentiated using a serological test to detect specific anti-N antibodies (Capua *et al.*, 2003). The same strategy was used to control H7N3 LPAI in Italy in 2002–2003 (Capua & Alexander, 2004),

in this case with a H7N1 vaccine. In both cases, vaccination combined with stamping out, using the described DIVA strategy resulted in eradication of the field virus. Problems with this system would arise if a field virus emerges that has a different N antigen to the existing field virus or if subtypes with different N antigens are already circulating in the field.

Alternatively the use of vaccines that contain only HA, e.g. recombinant vaccines, allows classical AGID and nucleocapsid protein (NP)- or matrix-based ELISAs to be used to detect infection in vaccinated birds. For inactivated vaccines, a test that detects antibodies to the nonstructural virus protein has been described (Tumpey *et al.*, 2005). This system is yet to be validated in the field.

5. Continued evaluation and updating of vaccine seed strains to protect against emergent variant field virus strains

Historically, H5 LPAI inactivated vaccine seed strains and recombinant fowl poxviruses with H5 gene inserts have shown broad cross protection in chickens against challenge by diverse H5 HPAI viruses from Eurasia and North American (Swayne & Kapczynski, 2008a). However, influenza A vaccines for poultry have had limited use in the field until 1995 when the H5N2 HPAI outbreak occurred in Mexico and vaccine use was implemented as part of the control programme (Villareal 2007). The HPAI strains were eradicated by June 1995, but as H5N2 LPAI viruses have continued to circulate, vaccination was maintained as one of the control tools for these H5N2 LPAI strains. Within a few years, multiple lineages of antigenically variant H5N2 LPAI field viruses emerged that escaped from immunity induced by the original 1994 vaccine seed strain used in the conventional inactivated vaccine (Lee *et al.*, 2004). Similarly, emergent H5N1 HPAI field viruses have arisen in China (the People's Rep. of), Indonesia and Egypt since 2005 that escaped from immunity induced by classical H5 inactivated vaccine seed strains used in commercial vaccines (Chen & Bu, 2009; Grund *et al.*, 2011; Swayne & Kapczynski, 2008b). It is not clear whether the emergence of these antigenic variants is related to use of vaccines or improper use of vaccines.

All influenza A vaccination programmes should have an epidemiologically relevant surveillance programme to check for emerging variants and representative isolates should be assessed for genetic and antigenic variation. Screening can be done by HI testing using genetic variant field viruses and vaccine seed strains as antigen, and isolates suspected of being antigenic variants should then be analysed by methods to quantify antigenic changes, e.g. antigenic cartography (Fouchier & Smith, 2010). Vaccine seed strains of HPAI and H5/H7 LPAI used in inactivated vaccines and recombinant vaccine viruses with H5 or H7 haemagglutinin gene inserts should be re-evaluated and seed strains that are not protective should be discontinued: a) whenever there is evidence of emergence of antigenic variants or vaccine failure (clinical disease in vaccinated flocks with a solid immune response to the vaccine antigen); or b) every 2–3 years for efficacy against circulating field viruses, and the use of seed strains that are not protective should be discontinued. The vaccine seed strain evaluation should include field viruses from all relevant geographical regions and production sectors, and sequence analyses of such viruses to identify genetic variants that can be further evaluated for antigenic change that may reduce the efficacy of the vaccine(s) in use. Strains representative of the major circulating antigenic lineage(s) plus selected antigenic variants should be used in challenge trials against current licensed vaccine seed strains, as well as potential future seed strains. Based on this scientific information, the competent veterinary authority within the country should establish, in consultation with leading veterinary vaccine scientists and international organisations, naturally isolated or reverse genetics LPAI vaccine seed strains for conventional inactivated vaccines, and H5 and H7 haemagglutinin gene insert cassettes for recombinant vaccines. In some situations, more than one seed strain may be necessary to cover all production sectors within a country. Only high quality and potent vaccines should be licensed and used in control programmes. Proper administration of high quality, potent vaccines is critical in inducing protective immunity in poultry populations.

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NB: There are OIE Reference Laboratories for Avian influenza
(see Table in Part 4 of this *Terrestrial Manual* or consult the OIE Web site for the most up-to-date list:
<http://www.oie.int/en/our-scientific-expertise/reference-laboratories/list-of-laboratories/>).
Please contact the OIE Reference Laboratories for any further information on
diagnostic tests, reagents and vaccines for avian influenza

APPENDIX 2.3.4.1.

BIOSAFETY GUIDELINES FOR HANDLING HIGHLY PATHOGENIC AVIAN INFLUENZA VIRUSES IN VETERINARY DIAGNOSTIC LABORATORIES

INTRODUCTION

The spread of highly pathogenic H5N1 avian influenza throughout Asia, Africa and Europe has led to an increase in the number of laboratories performing diagnostics for this pathogen. Highly pathogenicity avian influenza (HPAI) viruses, in general, are a serious threat to birds and mortality is often 100% in susceptible chickens. In addition, the agents can also pose a serious zoonotic threat, with over 50% mortality reported in humans infected with H5N1 HPAI virus. In recognition of the need for guidance on how to handle these viruses safely, the OIE has established the following biocontainment level guidelines for handling specimens that may contain high pathogenicity influenza A virus. They are based on biosafety guidelines published in this OIE *Terrestrial Manual* (2012) and the World Health Organization (2005).

BIOCONTAINMENT LEVELS

Samples for diagnostic testing for high pathogenicity influenza A virus using the following techniques can be processed using the OIE containment level for group 2 pathogens:

- Reverse transcriptase polymerase chain reaction (RT-PCR) and real-time reverse transcriptase polymerase chain reaction (real-time RT-PCR)
- Antigen-capture assays
- Serology

Virus isolation and identification procedures for handling specimens that may contain high-titred replication-competent HPAI virus should be performed at the OIE containment level for group 3 or group 4 pathogens, which would include the following:

- Personnel protective equipment should be worn, including solid-front laboratory coats, gloves, safety glasses and respirators with greater than or equal to 95% efficiency.
- Specimens from potentially infected birds or animals should only be processed in type II or type III biological safety cabinets (BSC).
- Necropsies of birds should be performed in a Type II BSC while wearing respiratory protection, such as a N95 respirator, or in a Type III biological safety cabinet, or other primary containment devices with 95% efficient air filtration.
- Centrifugation should be performed in sealed centrifuge cups.
- Centrifugation rotors should be opened and unloaded in a BSC.
- Work surfaces and equipment should be decontaminated after specimen processing.
- Contaminated materials should be decontaminated by autoclaving or disinfection before disposal or should be incinerated.

If chickens or other birds or mammals are inoculated with HPAI viruses, inoculation should be done in a containment level for group 4 pathogens and should include:

- Inoculated chickens should be held in isolation cages or other primary containment devices, or non-isolation cages/floor pens in specially designed rooms such as biosafety level 3 agriculture (BSL-3Ag) as designed by the US Department of Agriculture.
- Cages should be in a separate facility that is equipped to handle containment level for group 3 pathogens.

- The room should be under negative pressure to the outside and the cages should be under negative pressure to the room.
- Cages should have HEPA-filtered inlet and exhaust air.
- Biosafety cabinet or other primary containment devices should be available in the animal facility to perform post-mortem examinations and to collect specimens.

REFERENCES

WORLD ORGANISATION FOR ANIMAL HEALTH (OIE) (2014). *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals*. www.oie.int

WORLD HEALTH ORGANISATION (WHO) (2005). WHO laboratory biosafety guidelines for handling specimens suspected of containing avian influenza A virus, 12 January 2005.

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